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**Shifted T-cell polarization after agricultural dust exposure in mice and men.**

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## **ABSTRACT**

A low prevalence of asthma and atopy has been shown in farmers and agricultural workers. However, in these workers, a higher prevalence of respiratory symptoms has been reported, in which Th1- and/or Th17-responses may play a role.

We investigated the effect of exposure to dust extracts (DE) from different farms on airway inflammation and T-cell polarization in a mouse model and assessed T-cell polarization in agricultural workers from the same farms.

DE were prepared from settled dust collected at cattle and pig farms and bulb and onion industries. Mice were exposed to PBS, DE, house dust mite (HDM) or HDM+DE via nasal instillation, four times per week during five weeks. Hyperresponsiveness, pulmonary inflammation, IgE levels and T-cell polarization were assessed. Th(elper)-cell and Tc(ytotoxic)-cell subsets were investigated in peripheral blood samples from 33 agricultural workers and nine non-exposed controls.

All types of DE induced high levels of IL-17, IL-1 $\beta$  and IL-6 in mouse lung homogenates. DE-exposed mice had more mixed inflammatory infiltrates in the lungs, and more neutrophils compared to PBS-exposed mice. All types of DE protected against the HDM-induced Th2 response and methacholine hyperresponsiveness. Interestingly, occupationally exposed humans had higher frequencies of Th-cells spontaneously expressing IL-17 and IFN $\gamma$  compared to controls.

This study reveals that chronic exposure to different types of farm dust induces a Th/Tc-17 inflammatory response in mice and agricultural workers. This may contribute to the low prevalence of Th2-related diseases but may constitute a risk for other chronic respiratory diseases.

Key question: What is the effect of chronic exposure to farm environments on T cell subsets and lung inflammation in mice and men?

Bottom line: Chronic exposure to farm environments may protect against Th2 responses but induces Th17 responses in mice and men.

Why read on: This study reveals that chronic exposure to different types of farm dust induces a Th17 inflammatory response in mice and agricultural workers, which may contribute to the protection against Th2-related diseases but may increase the risk for other chronic respiratory diseases.

## INTRODUCTION

Several studies have shown that farmers and agricultural workers have a high prevalence of respiratory symptoms related to chronic bronchitis and chronic obstructive pulmonary disease.[1-3] However, it is well recognized that exposure to farm environments has a protective effect against the development of Th2-related allergic diseases.[4-7] Dose-response relations have been described for occupational endotoxin exposure in association with protection from atopic sensitization[[8-10] and allergic asthma,[11] whereas positive associations with increased airway responsiveness [8,9] and non-allergic asthma have also been reported.[11]

In mice, exposure to a mixed farm dust extract from cattle and goat stables protected mice from the development of ovalbumine (OVA)-induced asthma features.[12] Moreover, a shift in the T-cell response towards Th1 and Th17 subsets has been observed in mice after repetitive exposure to dust extract derived from pig confinement.[13] In humans, exposure to swine confinement induced levels of IL-17 and IL-17-expressing lymphocytes in bronchoalveolar lavage (BAL) fluid.[14,15] Interestingly, the Th17 response has been shown to be associated with the development of non-allergic or neutrophilic asthma in transgenic mice overexpressing ROR $\gamma$ t.[16] Hence, a shifted T-cell response could underlie the observed paradoxical effect of exposure to the farm environment in mice and humans.

Depending on the agricultural sector, the work-related microbial exposure can vary greatly.[11,17] However, to date, the effects of agricultural dust exposures from diverse sources on T-cell differentiation and the inflammatory response are unclear. Therefore, we determined whether dusts from two farms (cattle and pig) and two agricultural related environments (onion and bulb industry) changed T-cell responses and the inflammatory profile in mice. A clinically relevant house dust mite (HDM) murine model of experimental asthma was used to investigate the protective effect of different types of farm dust exposure on the HDM-induced allergic airway inflammation. In addition, human Th-cell subsets were assessed in occupationally exposed subjects from the same environmental settings as the dust used in the mouse experiments and compared to non-exposed subjects.

## MATERIAL AND METHODS

### Animal study design

Specific pathogen free female BALB/c mice (n=8 per experimental group, 8 week old) were anesthetized with isoflurane and intranasally exposed to each different dust extract (DE; details below; DE exposure: 1mg/ml in PBS, 50  $\mu$ l/day), house dust mite (HDM exposure: 40  $\mu$ g/day in 50  $\mu$ l PBS) or PBS (50  $\mu$ l/day) as a control, four times per week, during five consecutive weeks. To investigate the protective effect of dust exposure, mice were exposed to the combination of HDM and each type of DE. See online supplement (figure S1). Mice had access to standard food and water *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Permit Number: 4788E).

### Dust extracts

Settled dust was collected from farms, and agricultural industries in the Netherlands, and was extracted applying a protocol adapted from Peters *et al.*[12] For details, see online supplement. The endotoxin concentrations of the different DE were assessed using the limulus amebocyte lysate assay (Lonza) as described previously.[18] Fungal extracellular polysaccharide (EPS) levels were measured using a sandwich ELISA.[19] Observed LPS and EPS levels are presented in Table 1.

Table 1: Endotoxin and extracellular polysaccharide (EPS) levels measured in the different types of farm dust extracts at 1  $\mu$ g/ $\mu$ l.

Type of farm dust	Endotoxin (EU/ml)	EPS (EPS units/ml)
Flower bulb	11,872	2,330
Onion	15,346	420
Cattle	20,101	278
Pig	31,269	2,240

### House dust mite

House dust mite (HDM) consisted of crushed whole bodies from *Dermatophagoides pteronyssinus* (Greer Laboratories). Endotoxin content according to manufacturer: 12.05 EU/mg.

### **Assessment of methacholine (MCh) responsiveness**

One day after the last exposure, methacholine responsiveness was assessed as changes in enhanced pause (Penh) in conscious, spontaneously breathing animals using a whole-body plethysmography system (Buxco Electronics) as described previously.[20] The concentration of MCh inducing a 200% increase in Penh (PC200) was calculated.

### **Tissue collection**

After 5 weeks of exposure, mice were anaesthetized and sacrificed by cardiac exsanguination. Serum was collected and kept in -20 °C until use. The left lung lobe was snap frozen and kept in -80°C until preparation of lung homogenates for cytokine analysis. For details, see online supplement. Right lung lobes were carefully inflated with 0.9 ml 50% Tissue Tek, O.C.T. (Sacura) in PBS, and three of the of the four lobes were snap frozen and stored at -80°C until use, while the smallest right lung lobe was zinc-fixed and embedded in paraffin.

### **Measurement of HDM specific serum IgE**

House-dust mite-specific IgE was measured in serum as described previously.[21]

### **Cytokines**

Concentrations of IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-13 and IL-17 were measured in lung homogenates by 6-plex ELISA system (Affimetrix), or standard ELISA (eBioscience) for interferon (IFN) $\gamma$ .

### **Semi quantitative evaluation of inflammatory infiltrates**

Sections (3  $\mu\text{m}$ ) of zinc-fixed and paraffin-embedded lung tissue were stained for Hematoxylin-eosin. Semi quantitative scores for degree of inflammation from 0 to 3 were determined by a blinded reviewer. For details, see online supplement and figure S2.

### **Immunohistochemistry**

Four- $\mu\text{m}$ - thick cryosections of lung tissue were stained with specific antibodies to determine the presence of T-cells, Th17, Tc17,  $\gamma\delta$  T-cells, B-cells, neutrophils and macrophages. For details, see online supplement. Eosinophils were determined with peroxidase enzyme histochemistry using diaminobenzidine (DAB, Sigma Aldrich) as substrate.

For quantitative analysis, neutrophils, Th17 and Tc17 cells were counted manually and numbers were corrected for the total area of lung tissue section as assessed by morphometric analysis using Aperio ImageScope viewing software 11.2.0.780.

### **Human study population**

T cell subsets in human subjects were studied in 33 non-smoking Dutch agricultural workers (pig and cattle farmers and onion and flower bulb industry workers), with known high dust and microbial exposure, derived from the follow up of the initial OMeGA study [17], and 9 non-exposed controls derived from the NORM cohort. In the NORM study, healthy never- and current-smokers were included and extensively clinically characterized (NCT00848406). 10 non-smoking subjects were randomly selected per work-environment from the follow up cohort (with exception of pig farmers of whom only 3 non-smokers were available) and 9 non-smoking non-exposed controls were matched for age and gender. Hereafter, farmers and agricultural industry workers will be referred to as “agricultural workers” and abbreviated as “AW”. The study protocol was approved by the institutional ethics committee and all participants gave their written informed consent.

### **Sample preparation and *in vitro* stimulation**



Peripheral Blood Mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation and were stored in aliquots in liquid nitrogen. After thawing, cells were washed and resuspended in warm RPMI1640 with 10% FCS and  $8-10 \times 10^5$  cells per well were stimulated overnight with 25ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich) and 1  $\mu$ g/ml calcium-ionophore (Ca-Io, Sigma-Aldrich) at 37°C, 5%CO<sub>2</sub>. Monensin (2  $\mu$ g/ml, Sigma-Aldrich) was added to each sample to inhibit cytokine secretion. As a control, one sample of each subject was cultured overnight in complete RPMI at 37°C, 5%CO<sub>2</sub> without stimulation. For details, see online supplement.

### **Immunofluorescent staining and flow cytometric analysis**

After stimulation the cells were washed in 2% BSA/PBS and counted.  $10^6$  cells were used for the flowcytometry staining. Flowcytometry data were analyzed using FlowJo software 7.6.5 (Tree Star Inc.). For details, see online supplement and figure S6.

### **Statistical analysis**

Data are presented as median unless stated otherwise. The nonparametric Kruskal-Wallis test was used to assess whether differences between groups existed, followed by a Mann–Whitney *U*-test for post-hoc analysis. Different DE exposures were compared to control, and HDM+DE exposed groups were compared to HDM-exposed group. All analyses were performed using GraphPad Prism 5.0, and differences were considered statistically significant at two-sided *p*-values less than 0.05.

## RESULTS

### DE exposure induces IL-17 in mouse lung tissue

To determine the effect of DE exposure on the immune response in murine lung, the presence of Th1 (IFN $\gamma$ ), Th2 (IL-4) and Th17 (IL-17A) cytokines was investigated in lung homogenates of mice exposed to different types of DE or to PBS as control. Mice exposed to all different types of DE had higher levels of IL-17 in the lungs as compared to PBS-exposed mice (figure 1C). DE exposure had no effect on IFN $\gamma$ - or IL-4 levels (figure 1A and B).

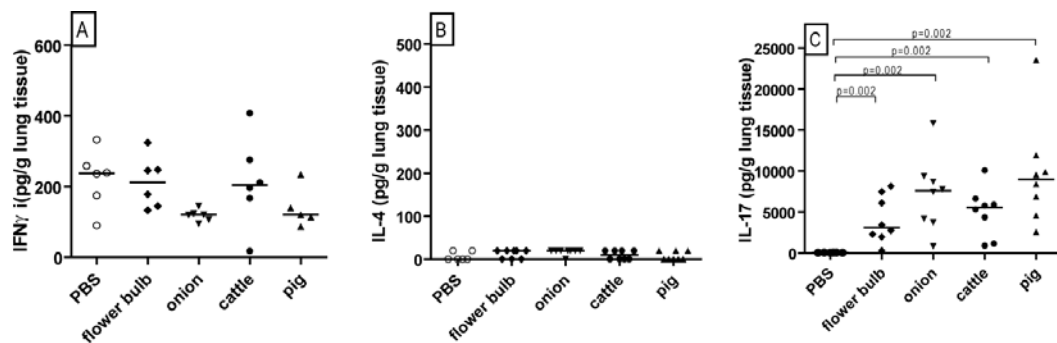


Figure 1: Cytokine levels in lung tissue. Levels of A) IFN $\gamma$ , B) IL-4 and C) IL-17 in lung tissue from mice exposed to PBS or DE derived from cattle or pig farms or flower bulb or onion industries (n=6-8 per group).

### DE exposure induces Th17 in mouse lung tissue

The source of IL-17 in lung tissue was investigated. All IL-17 positive cells were also positive for CD3, and thus of T-cell origin. Quantitative analysis revealed higher numbers of Th17 cells (CD4<sup>+</sup>/IL-17<sup>+</sup>, figures 2A and C) in the groups exposed to DE than in the PBS-exposed group. Tc17 cells (CD8<sup>+</sup>/IL-17<sup>+</sup>, figures 2B and D) showed a similar pattern although not statistically significant. IL-17 producing  $\gamma\delta$  T cells were not found (data not shown).

In addition, higher levels of IL-1 $\beta$  and IL-6, cytokines involved in Th17 and Tc17 differentiation, were found in lungs of DE-exposed groups as compared to PBS-exposed group (figure 2E and F).

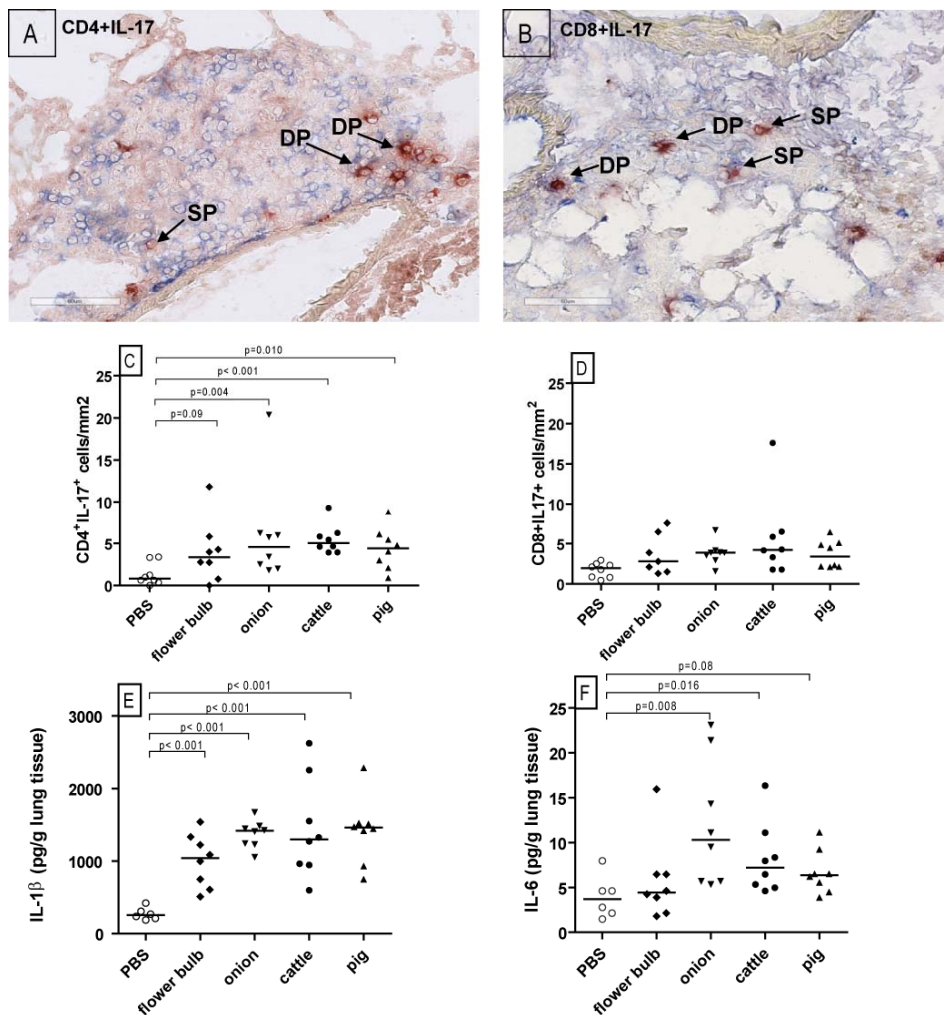


Figure 2: IL-17-producing T cells in lung tissue of mice exposed to DE from cattle farm are shown by immunohistochemical staining of A) CD4 (blue) and IL-17 (red), and B) CD8 (blue) and IL-17 (red) positive cells. Arrows indicate IL-17 single positive (SP) and double positive (DP) cells C) numbers of CD4IL-17 and D) CD8IL-17 double positive cells in lung tissue, E) IL-1 $\beta$  and F)IL-6 levels in lung tissue from mice exposed to PBS or DE from cattle or pig farm or flower bulb or onion industries (n=6-8 per group).

## DE exposure induces inflammatory infiltrates and neutrophilic inflammation in mouse lungs

All types of DE induced numerous mixed inflammatory infiltrates in mouse lungs (Figure 3A),

which were located perivascularly and peribronchiolarly, as well as in the parenchyma. Immunohistochemistry demonstrated these inflammatory infiltrates to be composed of T- and B cells, macrophages and neutrophils (figure S3). Quantification of neutrophils revealed that all types of DE induced lung neutrophilia (figure 3B).

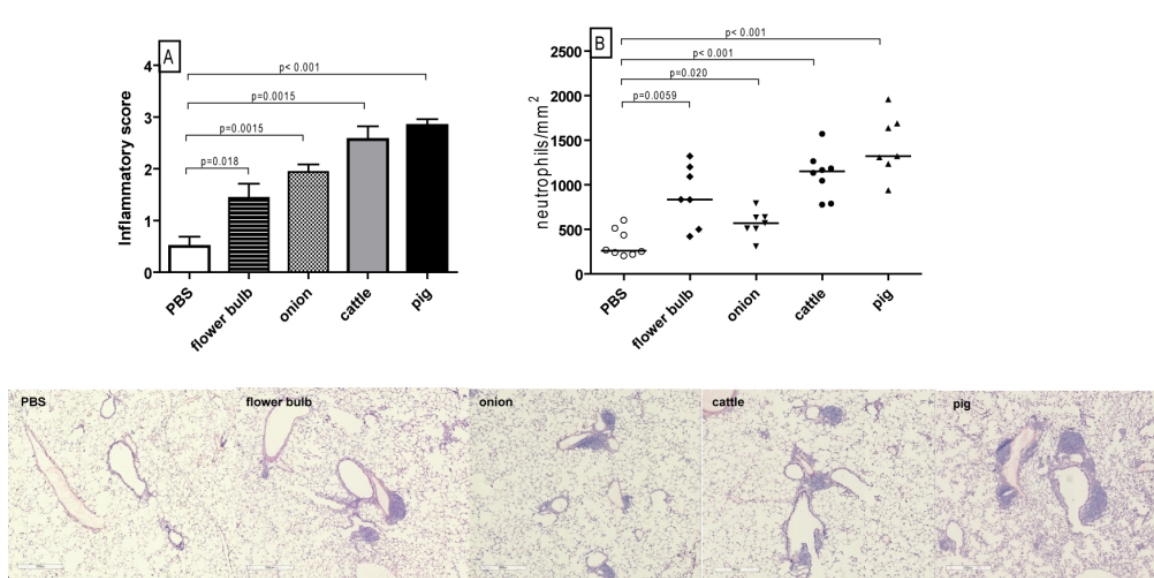


Figure 3: A) Inflammatory scores and B) numbers of neutrophils in lung tissue from mice exposed to PBS or DE from cattle or pig farm or flower bulb or onion industries (n=6-8 per group) Representative pictures of Hematoxylin-eosin stained lung sections of mice from each exposure group are shown.

### Exposure to DE does not increase MCh responsiveness

To assess whether exposure to DE induces changes in lung function, MCh responsiveness was measured. Exposure to all types of DE did not increase MCh responsiveness (figure S4).

### All DE protect against HDM-induced Th2 responses

The effect of different types of DE exposure on the HDM-induced Th2 response was determined. HDM exposure increased the levels of Th2 cytokines IL-4, IL-5 and IL-13 in lung tissue, but this increase was not observed in mice exposed to the combination of HDM + any type of DE (Figure 4 A-C). Similarly, numbers of eosinophils and levels of HDM-specific IgE were higher in HDM-exposed mice, while these HDM effects were not present in mice exposed to HDM+ each type of

DE (figure 4D,E). Mice exposed to HDM had a lower PC200, meaning increased MCh responsiveness, compared to PBS-exposed mice (figure 4F). This HDM-induced MCh responsiveness was prevented after DE exposure.

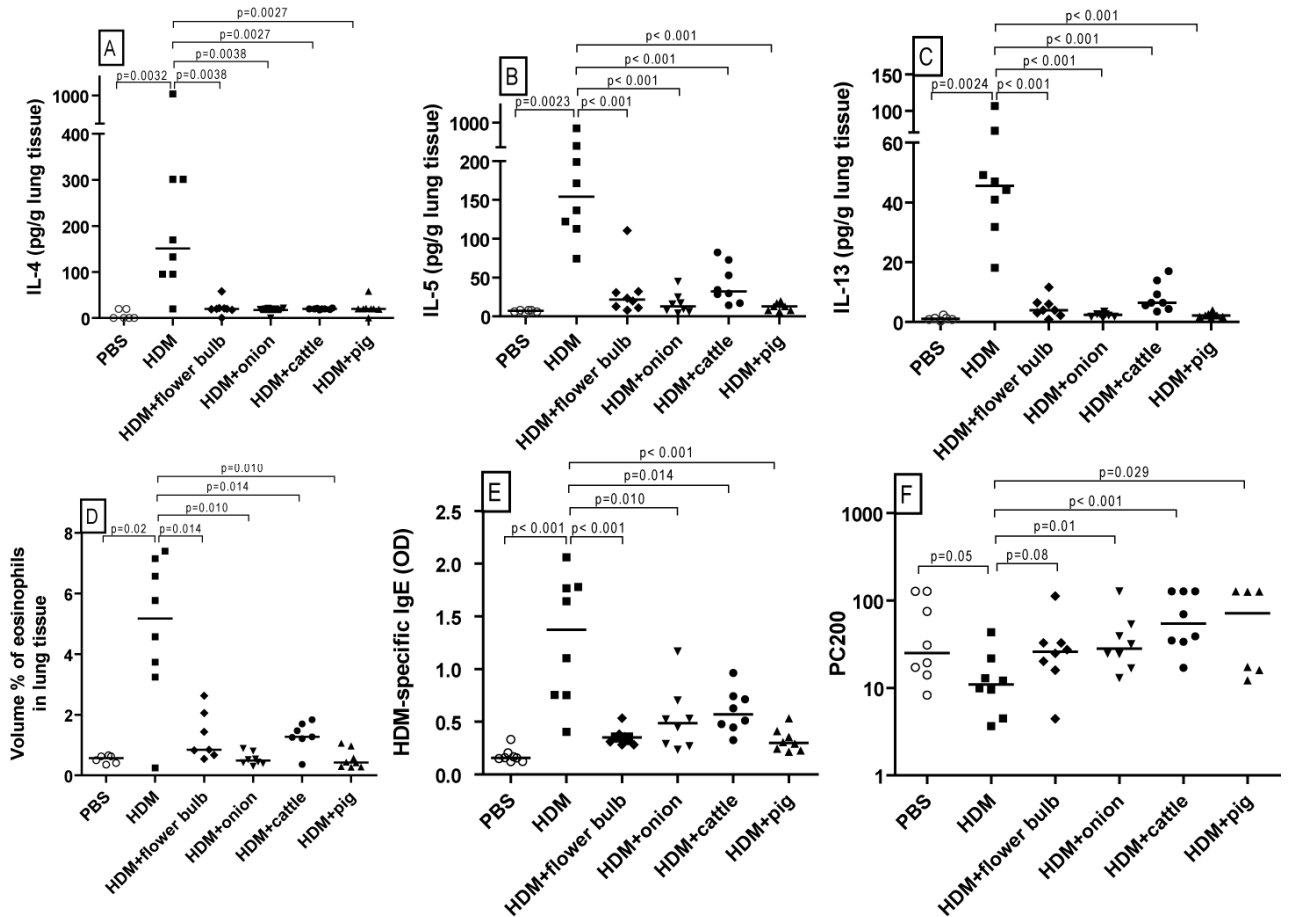


Figure 4: Levels of A) IL-4, B) IL-5, C) IL-13, D) eosinophils in lung tissue, E) HDM-specific IgE in serum and F) PC200 of mice exposed to PBS, HDM or HDM+DE derived from cattle or pig farms or flower bulb or onion industries (n=6-8 per group).

### Higher percentages of circulating Th17 and Th1 in agricultural workers

To translate our studies in mice to the human situation, the different Th-cell subsets were compared between agricultural workers (AW) from the same environmental settings as the dust used in the mouse experiments and non-exposed controls. Except for age, main characteristics of

the two study populations did not differ (Table 2). Main characteristics were similar for the four AW environments.

Table 2: Main characteristics of the study population

	Healthy Controls	Agricultural workers
Total number	9	33
Age (years)	35.5±20.8 (range 19-66)	46.5 ± 10.6 (range 29-72)
Male gender	9 (100%)	33 (100%)
Never smokers	9 (100%)	33 (100%)
BMI	24.7 ±4.5	25.5 ±3.0
FEV <sub>1</sub> % pred.	102.4% ±8.9	106.4%± 13.6
FVC % pred.	106.7% ±6.4	110% ±15.4

Values are given as mean ±SD unless otherwise indicated

Th-cell subsets were defined by simultaneous measurement of the intracellular presence of IL-4, IL-17 and IFN $\gamma$  in CD8<sup>-</sup> T-cells expressing the early activation marker CD69<sup>+</sup> and the presence of the transcription factor Foxp3 in CD8<sup>-</sup> activated (CD25<sup>+</sup>) T-cells. Please refer to figure S5 for gating strategies. Interestingly, AW had higher frequencies of CD8<sup>-</sup>CD69<sup>+</sup> T-cells spontaneously expressing IL-17 (Th17) or IFN $\gamma$  (Th1) than controls (figure 5 A and B, respectively). In addition, AW had lower percentages of IL-4<sup>+</sup> cells (Th2) within the CD8<sup>-</sup>CD69<sup>+</sup> T-cells as compared to controls (figure 5C). Since regulatory T-cells (Tregs) play an important role in the down regulation of Th1 and Th2 responses, we investigated the intracellular expression of Foxp3 within the CD8<sup>-</sup>CD25<sup>+</sup> T-cell population. There was no difference in frequencies of Tregs between AW and controls (figure 5D).

Stimulation with PMA/Ca-Io increased the frequencies of Th cells expressing IL-17 and IFN $\gamma$  in both AW and controls resulting in similar numbers of Th17 and Th1 cells for both groups (figure S7). IL-4 expression was not increased after PMA/Ca-Io stimulation and was similar in AW and controls (data not shown).

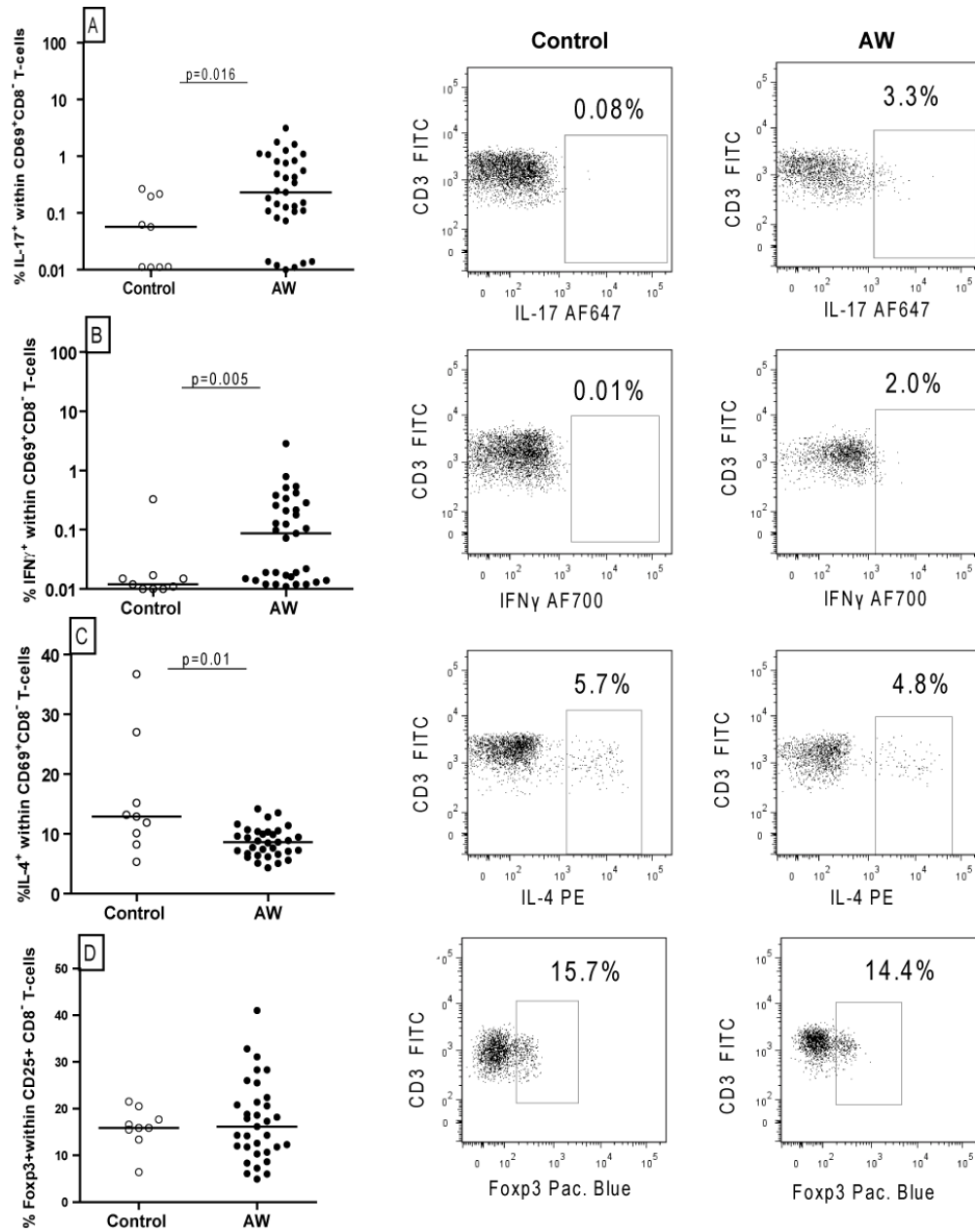


Figure 5: Percentages of circulating A) IL-17-, B) IFN  $\gamma$ - and C) IL-4-producing CD3<sup>+</sup>CD4<sup>+</sup>CD69<sup>+</sup> cells, and D) percentages of Foxp3-expressing CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells from controls and agricultural workers (AW). Representative dot plots of IL-17, IFN $\gamma$ , IL-4 expression in CD3<sup>+</sup>CD4<sup>+</sup>CD69<sup>+</sup> cells and Foxp3 expression in CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells from controls and AW are shown.

### Higher percentages of circulating Tc17 and Tc1 in agricultural workers

Similar to the findings in CD8<sup>+</sup> cells, AW had higher percentages of CD8<sup>+</sup> T-cells spontaneously expressing IL-17 (Tc17) and IFN $\gamma$  (Tc1) than controls (figure 6A and B). Percentages of IL-4<sup>+</sup> cells

within the CD8<sup>+</sup> T-cells were not different between both groups (data not shown). Overnight stimulation with PMA/Ionomycin increased the frequencies of CD8<sup>+</sup> cells expressing IL-17 and IFN $\gamma$  both in AW and controls resulting in similar levels for both groups (figure S7).

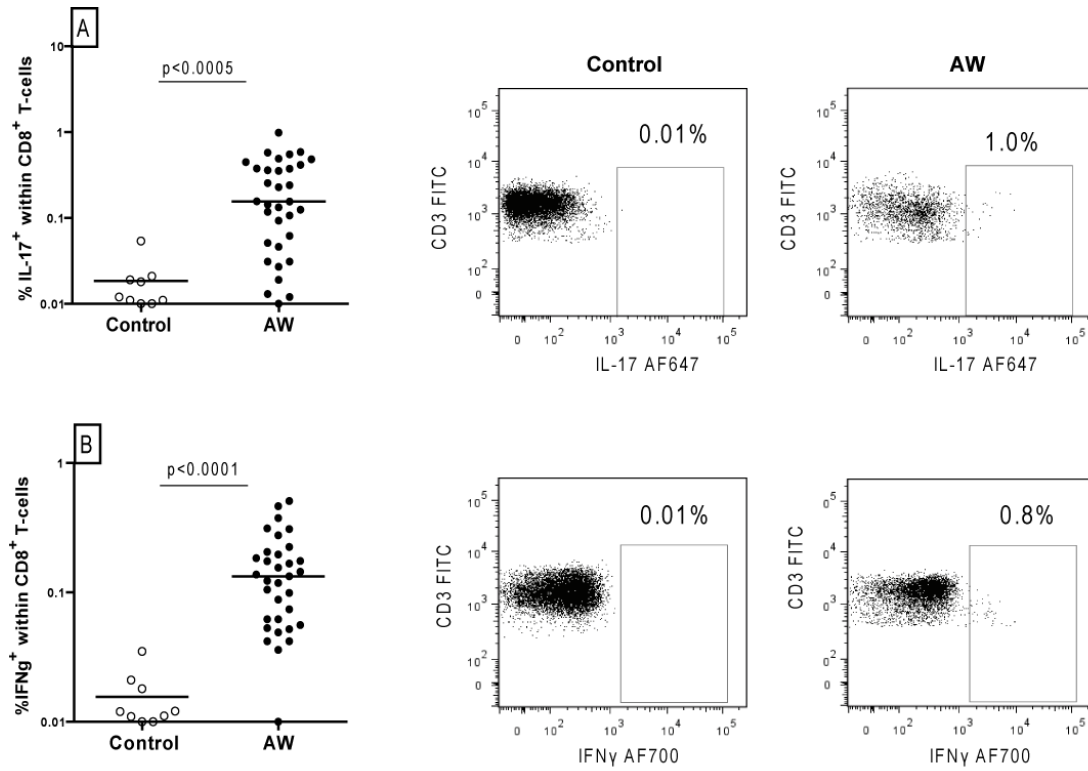


Figure 6: Percentages of A) IL-17- and B) IFN  $\gamma$ -producing CD3<sup>+</sup>CD8<sup>+</sup> cells from controls and agricultural workers (AW). Representative dot plots of IL-17 and IFN $\gamma$  expression in controls and AW are shown.



## DISCUSSION

This study demonstrates that chronic exposure to different types of DE induces Th17 responses accompanied by lung neutrophilia and mixed inflammatory infiltrates in mouse lungs. In addition, all types of DE protected against HDM-induced Th2 responses, i.e. airway eosinophilia, HDM-specific IgE in serum, and IL-4, IL-5 and IL-13 production in the lung tissue. Interestingly, our results from the mouse model are supported by the human data. Occupational organic dust exposure skewed the circulating T-cell population towards the Th17 and Th1 phenotypes compared to controls, while the Treg frequencies were of similar size.

Levels of occupational endotoxin exposure are known to vary greatly depending on the type of work environment or agricultural activity.[17] Previously, we observed a linear dose-response relationship between increased endotoxin exposure and a higher risk of respiratory symptoms, [17] which was dependent on level of exposure but seemed to be independent of work environment. In line with these findings, we show in mice that exposure to DE from four different agricultural settings induced Th17 differentiation and consequent lung inflammatory infiltrates as well as protection against Th2 responses. These settled dust samples are complex mixtures and it is difficult to identify causal agents without considering co-exposure to other relevant agents. In our study, as a result of the extraction procedure of the dust, a bias towards NaCl soluble components may have occurred.

Exposure of healthy individuals to dusts from agricultural environments has been shown to result in an intense inflammatory response, characterized by high numbers of neutrophils and lymphocytes in bronchial lavage.[14,22] This response seems to attenuate over time, but repetitive exposures may lead to chronic respiratory disease.[2] Since IL-17 is known to be involved in the IL-8-dependent attraction of neutrophils to the lung, we hypothesized that IL-17 is involved in the DE-induced inflammatory response. In this study, we showed that chronic exposure to different types of DE induced IL-17 production in murine lung tissue, which was accompanied by marked lung inflammation. This inflammation was characterized by neutrophilia, inflammatory aggregates, mainly composed of macrophages and B- and T- lymphocytes, and higher levels of additional inflammatory cytokines (IL-1 $\beta$  and IL-6). These findings are in

agreement with other mouse studies using pig DE.[23,24] However, the effect of DE from other types of farms have until now not been described. We show that exposure to other types of DE induced a similar type of inflammatory response as exposure to pig DE.

We demonstrated the presence of both Th17 and Tc17 cells in murine lungs after DE exposure. This indicates that these IL-17-producing T-cell subsets are important in host defense against microbial components present in DE, which is in line with previous studies.[25,26]. Other studies described IL-17 production by  $\gamma\delta$ T cells and neutrophils, [27],[28] which was not observed in our study.

The observed protective effects of DE in the HDM-induced asthma model support data obtained by previous studies in OVA mouse models for asthma. In these studies, exposure to dust extract,[12] polysaccharides,[29] and specific microbes [30-32] isolated from farm cowsheds also protected against OVA-induced allergic airway inflammation. Mechanisms underlying the observed allergy protection in these studies included upregulation of Th1 cells [30-32] or induction of tolerogenic dendritic cells,[12,29] as reflected by increased levels of IFN $\gamma$  and IL-10, respectively. IFN $\gamma$  levels were not increased after HDM+DE exposure compared to mice exposed to HDM alone, excluding upregulation of Th1 as the mechanism of protection in our model.

Hence, the mechanism of protection in our mouse model remains to be elucidated.

According to the hygiene hypothesis, microbial exposures during childhood are important for the development of a healthy Th1 response, to counterbalance the Th2-biased immune system of newborns.[33] More recent studies have shown that exposures to a farm environment protect from Th2 responses independently from farm childhood.[4] We show here that agricultural workers have higher percentages of circulating Th17 and Th1 cells, while the percentage of Th2 cells was lower than in non-exposed controls. These findings support the large amount of literature showing a lower prevalence of Th2-related allergic diseases in farmers and agricultural workers.[9,11,17] Besides the increased percentages of Th17 cells in agricultural workers, the percentages of Tc17 cells, a subset of CD8<sup>+</sup> T-cells that produces IL-17, were also increased. While a skewing of the immune system towards the Th1 subset seems to be protective against allergic diseases, the increase in circulating IL-17<sup>+</sup> cells in agricultural workers may be related to

the development of chronic respiratory lung disease and possibly related to respiratory infections.[25] Interestingly, levels of IL-17, IL-17-expressing cells were reported to be increased in human BAL fluid after acute inhalation of organic dust in a pig confinement.[14,15] Although these studies were performed with a low number of individuals (15 and 6, respectively), the induction of IL-17 in BAL indicates that a specific IL-17 response is induced by exposure to a farm environment. It should be acknowledged that no females and no smokers were included in our study. It is not known whether differences are to be expected in responses according to gender. As active smoking is known to affect the immune response, we have restricted our population to non-smoking individuals.

Interestingly, differences in T-cell subsets between agricultural workers and healthy controls were only found in unstimulated cells. These cells reflect the actual T-cell phenotype in the host, which indicates that circulating T-cells from agricultural workers are different from T-cells in healthy controls. After stimulation with PMA/Ca-*lo*, higher frequencies of cells expressing the early activation marker CD69 were found and within this population, higher frequencies of T-cells producing IFN $\gamma$  and IL-17. However, after this stimulation, no differences were found between agricultural workers and controls. This can probably be explained by the strong stimulation by the protein kinase C activator, PMA and the calcium ionophore, which could have induced a plateau in cytokine production in both groups.

In conclusion, this study shows that chronic exposure to various agriculture environments induces a shift in the T-cell population towards a Th17/Th1 phenotype. This may on the one hand protect from allergic disease development as shown in our mouse model, but on the other hand may contribute to other chronic lung disease, as suggested by the lung neutrophilia in the same mouse model. It is clear that the role of IL-17 and Th17 polarization in the agriculture dust-induced phenotype demands further investigation.

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## **CONTRIBUTIONS**

P. Robbe, E.A.J. Spierenburg, C.A. Brandsma, D. Heederik, W.Timens, B.N. Melgert, I.M. Wouters and M.N. Hylkema contributed to the design and planning of the experiments. P.Robbe, E.A.J. Spierenburg, C. Draijer, E.Telenga, , M van den Berge and M. Luinge conducted fieldwork and laboratory experimental work. P.Robbe, E.A.J. Spierenburg, C.A. Brandsma, D. Heederik, W.Timens, B.N. Melgert, I.M. Wouters, M.N. Hylkema, C. Draijer, E.Telenga, A.J.M. van Oosterhout, M van den Berge and M. Luinge contributed to the reporting of findings and writing the manuscript.

## **COMPETING INTERESTS**

The authors declare no competing interests.

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## ONLINE SUPPLEMENT

### METHODS

#### Animal study design

8 week old specific pathogen free female BALB/c mice (Harlan, Horst, The Netherlands) were housed in 10 groups of 8 and had access to standard food and water *ad libitum*. Female mice were chosen because, according to our previous study, female mice of this strain have a more prominent response to the HDM model of asthma. More specifically, female mice have more HDM specific IgE, more eosinophils in the lungs and are more responsive to methacholine than male mice.[1] Mice were anesthetized with isoflurane and intranasally exposed to each different dust extract (DE; details below; DE exposure: 1mg/ml in PBS, 50  $\mu$ l/day), house dust mite (HDM exposure: 40  $\mu$ g/day in 50  $\mu$ l PBS) or PBS (50  $\mu$ l/day) as a control, four times per week, during five consecutive weeks. To investigate the protective effect of dust exposure, mice were exposed to the combination of HDM and each type of DE. Mice had access to standard food and water *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Permit Number: 4788E). A volume of 50  $\mu$ l was chosen as studies on distribution of intranasal instillations in mice have shown that a maximum distribution to the lungs is achieved with this volume.[2]

#### Dust extracts

Settled dust was collected from 30 cattle and 7 pig farms, and from 2 flower bulb and 3 onions agricultural industries in the Netherlands and were pooled by type of agricultural setting. Pooled dust was extracted applying the protocol from Peters *et al* with some adaptations [3]. Briefly, aliquots of approximately 0.5 gram of settled dust samples collected at farm or in agricultural industries were pooled. Mixed dust samples for each location (cattle, pig, flower bulb and onion)

ranged between 2.5 and 7.5 gram of settled dust and were mixed with glass beads in a 1:5 ratio (dust:beads) in a 50 ml Greiner tube, ~2.5 gram dust/tube. Braun sterile water was added to a volume of 50ml. Samples were then mixed for 5min on end-over-end roller at room temperature and subsequently crushed for 5 minutes in ultrasonic bath with crushed ice and transferred to a glass Erlenmeyer. Then sterile water was added to a final concentration of 10 mg dust per ml. Sodium chloride solution was added to obtain a final concentration of 0.9% followed by shaking for 6 hours on horizontal shaker at room temperature. Then samples were centrifuged at 20.000g/4°C/45min and after centrifugation the dust extract was dialyzed (MWCO 3500) against di-distilled water, sterilized through filtration through a 0.22 um filter and lyophilized. Hereafter, the extracted dust from both farms and agricultural industries is referred to as 'DE'. Dose responses experiments have been performed by the group of Peters *et al.* [3] using similar extraction, in which optimal dose was determined to be 50µg/ day (personal communication).

### **Lung homogenates**

Snap frozen lung tissue was mechanically homogenized (50% w/v) in 50 mM Tris-HCl buffer, containing 150 mM NaCl, 0.002% Tween-20 (pH 7.5) and a protease inhibitor (Sigma Aldrich). Homogenates were centrifuged at 12000xg for 10 minutes to remove any insoluble material. Supernatants were subsequently stored at -80°C until further analysis.

### **Semi quantitative analysis of inflammatory infiltrates**

Sections (3 µm) of zinc-fixed and paraffin-embedded lung tissue were stained for Hematoxylin-eosin. Semi quantitative scores for degree of inflammation from 0 to 3 (the greater the score, the greater the inflammatory infiltration in the lung) were determined by a blinded reviewer.

Evaluation of the whole lung tissue present in each slide included the bronchiolar compartment as well as the parenchyma. For representative pictures of each inflammatory score, see figure S2.

### **Immunohistochemistry**

To determine the presence of T cells, B cells, neutrophils and macrophages, 4 µm-thick cryosections of lung tissue were stained with hamster anti-mouse CD3 (BD Bioscience), rat anti-mouse CD19 (BD bioscience), rat anti-mouse GR1 (BD Bioscience) and rat anti-mouse CD68 (AbD serotec) antibodies respectively. To determine the presence of Th17 and Tc17 cells, lung cryosections were stained with a monoclonal rat anti-mouse IL-17 antibody (*R&D systems*) in combination with a monoclonal hamster anti-mouse CD3 antibody (*BD Biosciences*), a monoclonal rat anti-mouse CD4 antibody (*BD Pharmingen*), a monoclonal rat anti-mouse CD8 antibody (*BD Pharmingen*), or a monoclonal hamster anti-mouse γδ antibody (*BD Pharmingen*).

### **Sample preparation and *in vitro* stimulation**

Peripheral Blood Mononuclear cells (PBMCs) were isolated by standard protocols using Lymphoprep for AW and Ficoll-Paque density gradient centrifugation for healthy controls. Agricultural workers' PBMC were stored in aliquots of  $3 - 7 \times 10^6$  cells in liquid nitrogen in a solution of 10% DMSO in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) supplemented with 50U/ml Penicillin/Streptomycin, 10% FCS and 0.1% β-Mercapto-ethanol (Merk, Darmstadt, Germany) until use.  $5-10 \times 10^6$  cells from healthy controls were stored in liquid nitrogen in a solution of 10% Dimethylsulphoxide (DMSO, Sigma-Aldrich, Steinheim, Germany), 50% FCS (Invitrogen, New York, USA) and 40% Hank's Balanced Salt Solution (HBSS, Lonza, Basel, Switzerland). Both isolation and freezing methods were compared and showed difference in PBMC yield, but no influence on T-cell populations (data not shown). Cells were quickly thawed in a water bath and slowly diluted in warm RPMI1640 with 10% FCS to dilute the DMSO avoiding osmotic stress. After washing, cells were resuspended in warm RPMI1640 with 10% FCS and  $8-10 \times 10^5$  per well were stimulated overnight with 25ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, Steinheim, Germany) and 1 µg/ml calcium-ionophore (Ca-Io, Sigma-Aldrich, Steinheim, Germany) at 37 degrees Celcius, 5%CO<sub>2</sub>. Monensin (2 µg/ml, Sigma-Aldrich, Steinheim, Germany) was added to each sample to inhibit cytokine secretion. As a control, one sample of each subject was cultured overnight in complete RPMI at 37°C, 5%CO<sub>2</sub> without stimulation.

### **Immunofluorescent staining**

After stimulation the cells were washed in 2% BSA/PBS and counted.  $10^6$  cells were used for the flowcytometry staining and incubated with 0.5% human serum (Lonza, Breda, The Netherlands) to block nonspecific bindingsites. Then, cells were washed and incubated with an antibody cocktail containing FITC-conjugated anti-CD3 (BD Biosciences, Breda, the Netherlands), Krome orange- conjugated anti-CD8 (Beckman Coulter, Woerden, the Netherlands), PECy7-conjugated anti-CD25 (eBioscience, Vienna, Austria) and APCCy7-conjugated anti-CD69 (Biolegend, San Diego, USA) for 30 min in the dark on ice.

Following membrane staining, cells were fixed and permeabilized (Fix/Perm buffer, eBioscience, Vienna, Austria) and washed with cold permeabilization buffer. To block nonspecific bindingsites, cells were incubated for 15 min. with 2% human serum, followed by an antibody cocktail containing Pacific Blue-conjugated anti-Foxp3 (Biolegend, San Diego, USA), PE-conjugated anti-IL-4 (eBioscience Vienna, Austria), Alexa Fluor 647-conjugated anti-IL-17 (eBioscience, Vienna, Austria) and Alexa Fluor 700-conjugated anti-IFN $\gamma$  (eBioscience, Vienna, Austria). After incubation for 30 min in the dark on ice, cell suspensions were washed twice with cold permeabilization buffer and resuspended in fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences, Breda, the Netherlands). Multi-color fluorescent staining was immediately measured on a LSR-II flow cytometer (BD Biosciences, Breda, the Netherlands) and analyzed using FACS Diva software (BD Biosciences, Breda, the Netherlands).

### **Flow cytometric analysis**

Flowcytometry data were analyzed using FlowJo software 7.6.5 (Tree Star Inc., Ashland, USA). Because stimulation reduces surface expression of CD4 on T cells, CD4 $^+$  T cells were identified indirectly by gating on CD3 $^+$  and CD8 $^-$  lymphocytes. Positively and negatively stained populations were calculated by dot plot analysis, as determined by unstained samples. The appropriate isotype controls for the cytokine (IL-4, IL-17 and IFN $\gamma$ ) staining (mouse IgG1 labeled with PE, AF647, and AF700 respectively) are represented in figure S6.

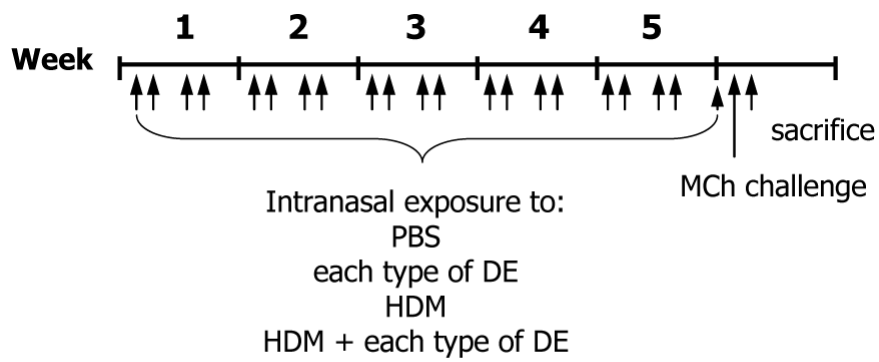


Figure S1 online supplement: Schematic overview of the experimental design

Figure S2 online supplement: Pictures of for Hematoxylin-eosin staining of lung tissue of mice exposed to PBS or DE representing the different degrees of inflammatory scores.

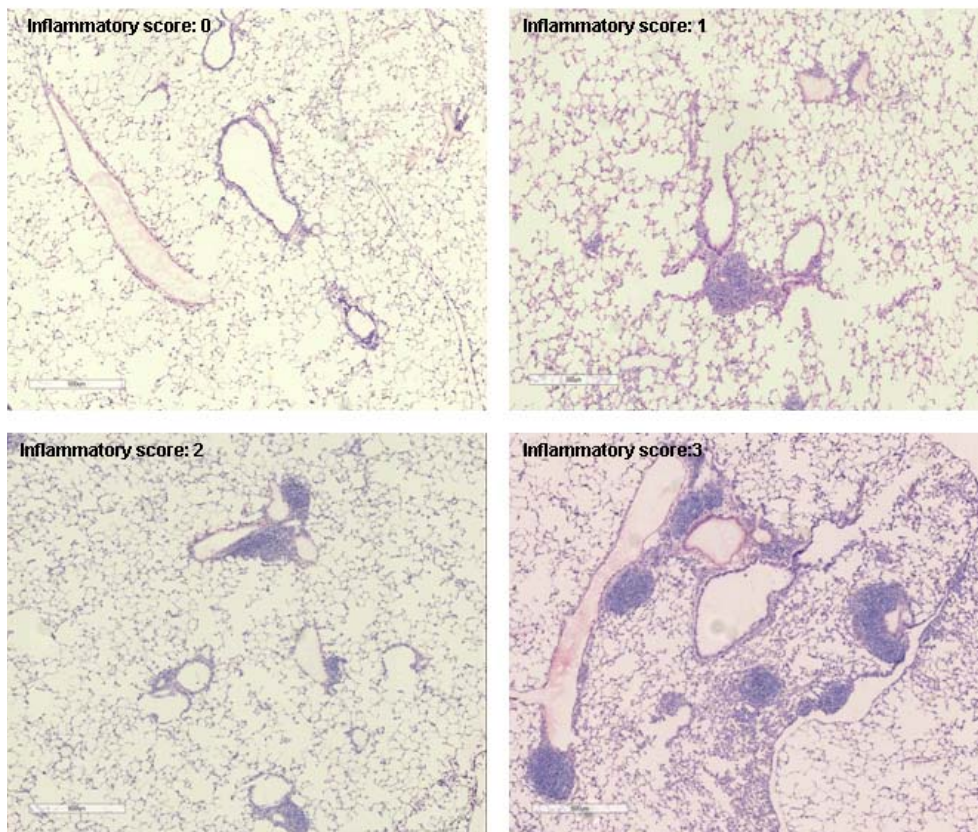
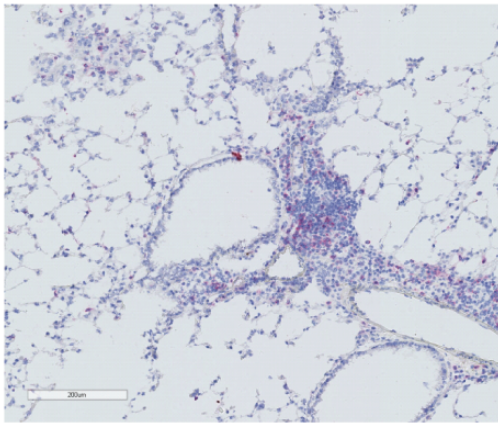
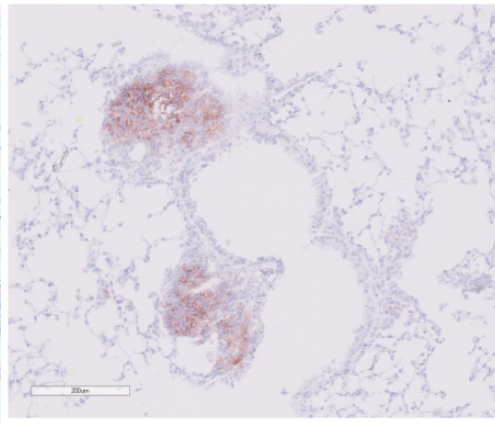


Figure S3 online supplement: Representative pictures of immunohistological staining with anti-CD3 (T-cells), anti-CD19 (B-cells), anti-CD68 (macrophages) and anti-GR-1 (neutrophils) in lung tissue from mice exposed to DE from cattle farms.

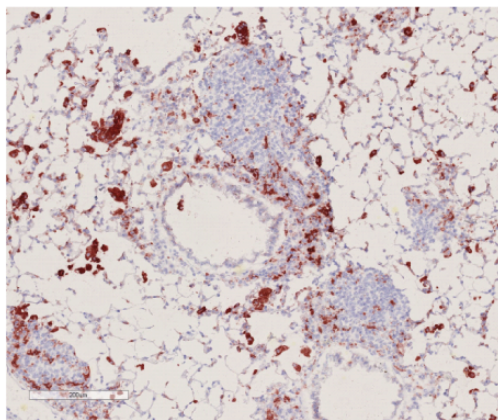
T cells



B cells



Macrophages



Neutrophils

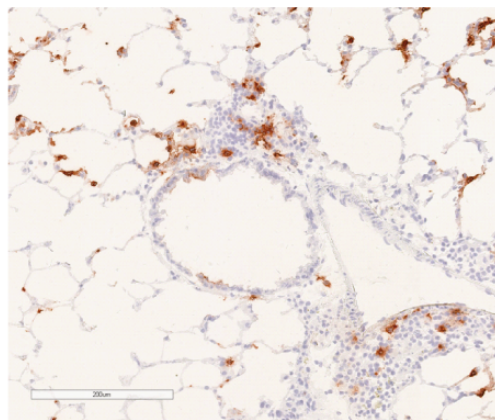




Figure S4 online supplement: Methacholine responsiveness from mice exposed to PBS or DE

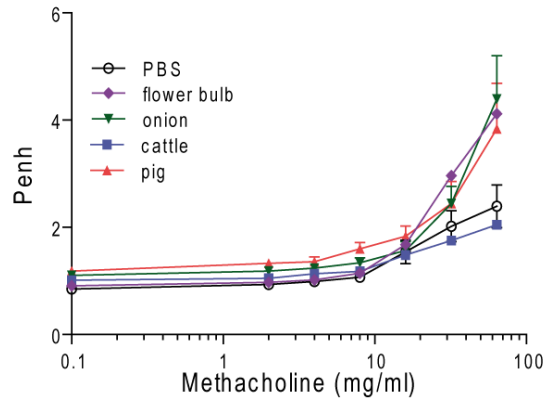


Figure S5 online supplement: Gating strategies to distinguish T-cell populations. FSc-SSc profile was used to distinguish lymphocytes. Within the lymphocyte population, T lymphocytes were identified using the membrane marker CD3, and within the CD3 population, Tc cells were identified using the marker CD8. The CD3<sup>+</sup> cells which were negative for CD8 were considered as being CD4<sup>+</sup> cells. Within the CD8<sup>+</sup> population, a CD69<sup>+</sup> population was selected and in this population the expression of the cytokines (IL-4, IL-17 and IFN $\gamma$ ) was analysed.

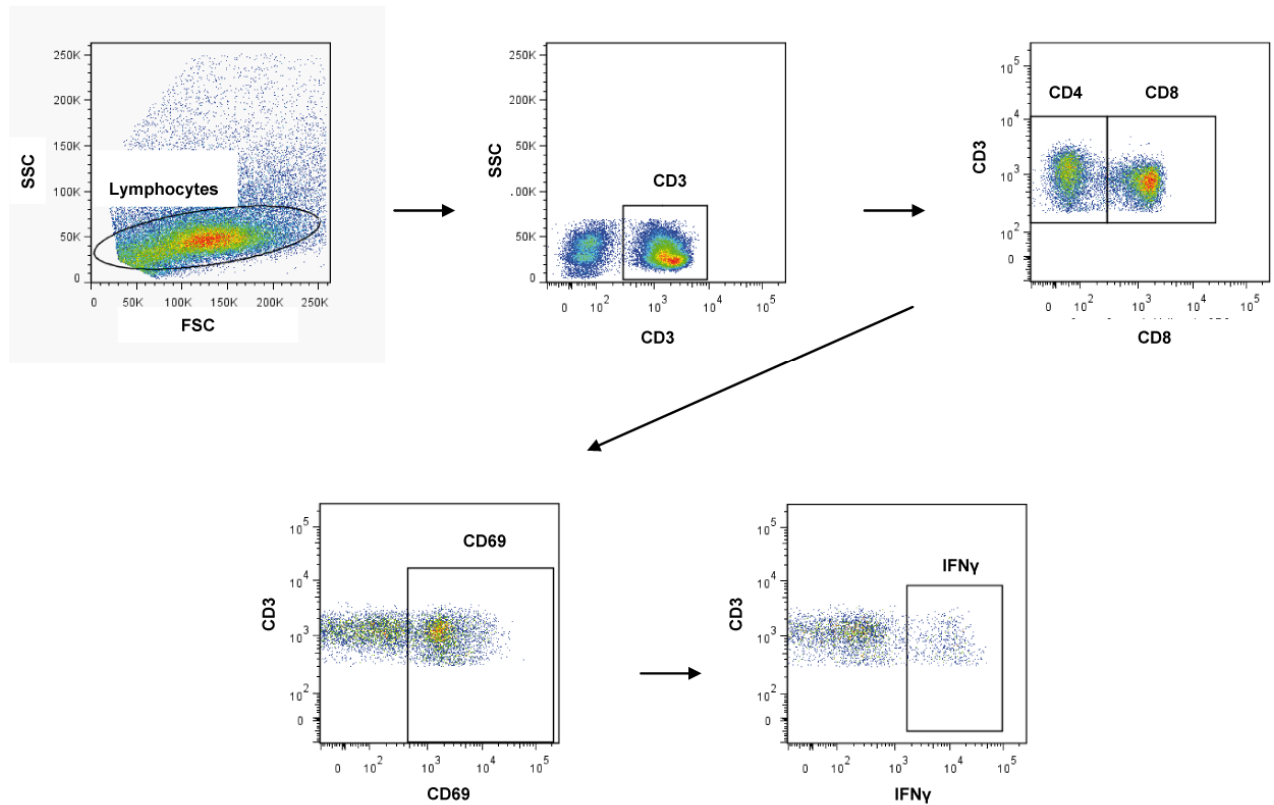




Figure S6 online supplement: Histograms of samples stained for IL-17, IFN $\gamma$ , and IL-4 (right column) and the respective isotypes (left column).

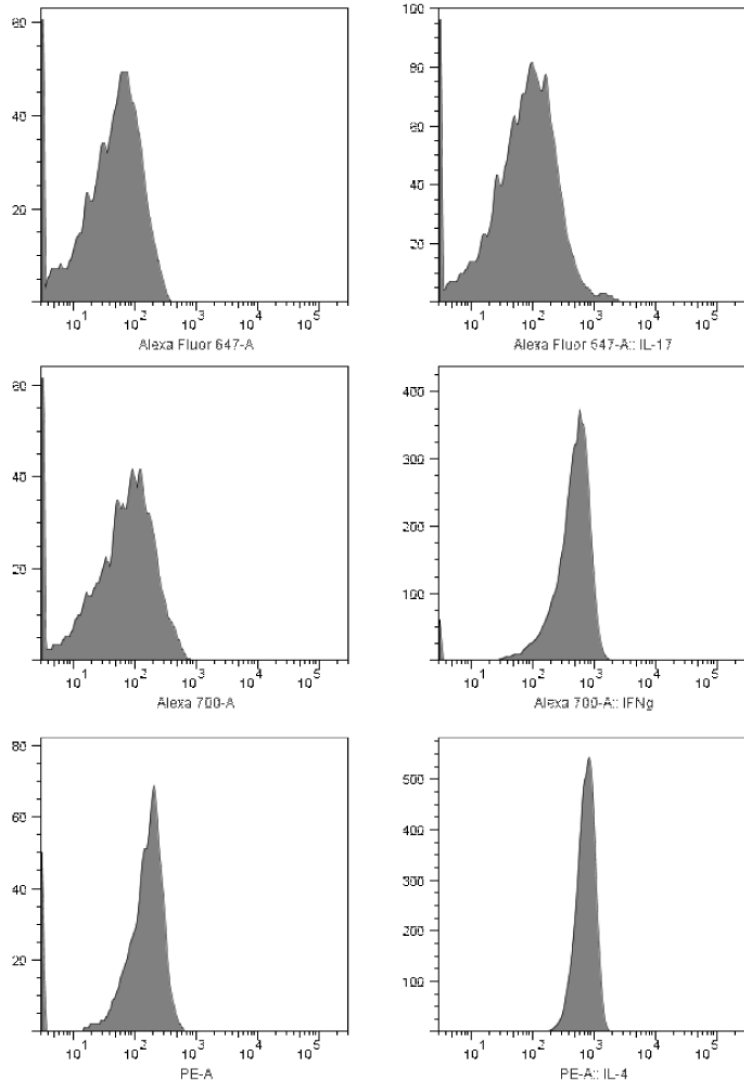
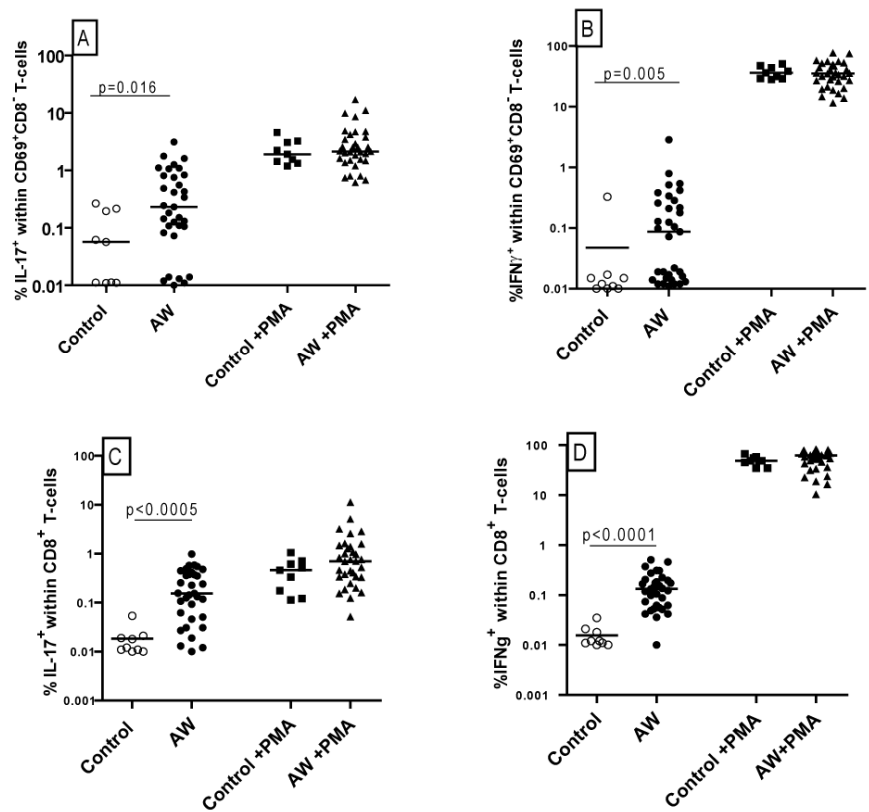


Figure S7 online supplement: Percentages of A) IL-17- and B) IFN $\gamma$ -producing T-helper and C) IL-17- and D) IFN $\gamma$ -producing T-cytotoxic cells with and without stimulation with PMA/Io. in controls and AW.



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